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The objective of this project has been to investigate three prejunctional mechanisms that may modulate acetylcholine (ACh) release at the motor nerve terminal of skeletal muscle: 1) prejunctional nicotinic cholinoceptor regulation of release, 2) modulation through preconditioning patterns of nerve stimulation, and 3) precursor control. In addition, the influence of acetylcholinesterase (AChE) inhibition on these mechanisms has been examined. Neuromuscular transmission has been assessed in the vascular perfused rat phrenic nerve-hemidiaphragm preparation (VPRH) by measuring the release of ACh

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directly by radioenzymatic assay or a newly developed chemiluminescent assay and indirectly by intracellular recordings and force of contraction measurements. Additional experiments have been done on rat sciatic nerve in order to examine the axonal transport of nicotinic binding sites. The mouse hemidiaphragm preparation has been used to study antidromic activity (backfiring) in the phrenic nerve in the presence of anti-AChE agents. Pharmacologic experiments with several nicotinic agonists and antagonists indicate that motor nerve terminals are populated by nicotinic cholinoceptors which act to modulate the amount of ACh release. Receptor binding studies with $^{125}$I-alpha bungarotoxin suggest that these nicotinic receptors resemble nicotinic receptors of the central nervous system. These receptors, in addition to modulating ACh release, are also responsible for stimulus-induced antidromic activity (backfiring) in the phrenic nerve. Studies with calcium channel blockers and calcium-calmodulin inhibitors suggest a central role for calcium in the phenomenon of backfiring. Evidence has also been obtained that choline availability can influence ACh release. Low levels of choline decrease release. However, this modulatory mechanism appears to represent a long-term process as opposed to the impulse-to-impulse regulation afforded by prejunctional nicotinic receptors.


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## SUMMARY

The specific aims of this project were to investigate three mechanisms through which acetylcholine (ACh) release may be modulated prejunctionally at the motor nerve terminal of skeletal muscle. These mechanisms include: 1) prejunctional cholinoceptor regulation of ACh release, 2) modulation of ACh release through preconditioning patterns of nerve stimulation, and 3) precursor control of ACh release. Neuromuscular transmission was assessed in the vascular perfused rat phrenic nerve-hemidiaphragm (VPRH) preparation by measuring the release of ACh directly by radioenzymatic assay or by chemiluminescence assay, and indirectly by intracellular recordings and force of contraction (FC) measurements. Additional experiments have been done on rat sciatic nerve in order to examine the axonal transport of nicotinic binding sites. The mouse hemidiaphragm preparation has been used to study antidromic activity (backfiring) in the phrenic nerve in the presence of an anticholinesterase agent.

The potent nicotinic receptor agonist suberyldicholine ($10^{-5}$M) caused a significant decrease in stimulus-evoked (10 Hz) ACh release from VPRH. Similar effects were found with equimolar concentrations of nicotine and carbamylcholine. The natural agonist ACh provoked frequency-dependent negative feedback on subsequent release when the neuromuscular junction concentrations of ACh were raised with neostigmine or other acetylcholinesterase (AChE) inhibitors. FC was compromised in a dose- and frequency-dependent manner by procedures that provoke negative feedback. The nicotinic antagonists d-tubocurarine (dTC $10^{-7}$M and $10^{-8}$M) and alpha-bungarotoxin (aBuTX 50 ug) induced an increase in ACh release from the VPRH during stimulation. dTC appeared to antagonize negative feedback at low doses ($10^{-8}$M), as evidenced by a partial restoration of FC during agonist-induced negative feedback. Intracellular recordings from the VPRH demonstrated that the frequency dependence of partial dTC neuromuscular blockade is associated with a prejunctional mechanism. This mechanism is compatible with the action of dTC on a prejunctional cholinoceptor. These experiments ruled out the common theory that dTC exerts its blocking action via direct sodium channel blockade.

Antidromic action potentials (backfiring) in the mouse phrenic nerve as a result of AChE inhibition have been linked to a prejunctional mechanism requiring the release of ACh. dTC antagonized stimulus-induced backfiring in the presence of neostigmine, implicating ACh feedback on the nerve terminal. Rapid vascular perfusion of a concentrated bolus of ACh induced transient backfiring in the mouse phrenic nerve. Prevention of ACh release by Clostridium botulinum toxin or tetanus toxin eliminated backfiring and, thus, disproved the theory that backfiring is due to a direct action of the AChE inhibitor. Verapamil (5 uM), a calcium antagonist, decreased spontaneous antidromic activity induced by neostigmine, but did not decrease miniature endplate potential amplitude or frequency. These studies suggest that verapamil may be acting on a prejunctional site which decreases nerve excitability. Whether this site is directly involved in the action of ACh on the nerve terminal remains uncertain.

Further experimentation on prejunctional calcium mechanisms focused on the role of calmodulin in local modulatory mechanisms of neuromuscular transmission. Four putative calmodulin inhibitors, including three neuroleptic phenothiazines and one naphthalenesulfonamide compound, were shown to depress neuromuscular transmission. Since $Ca^{2+}$-calmodulin is thought to regulate neurotransmitter release via a cascading pathway involving adenylate cyclase, cAMP, and phosphorylation of a protein by a kinase, pharmacologic antagonism of the calmodulin inhibitors was explored with three compounds that elevate intracellular cAMP levels. The results revealed the predicted antagonism, thus supporting the theoretical cascading pathway in which $Ca^{2+}$-calmodulin regulates intraterminal cAMP levels which in turn modify ACh release.

Binding studies in sciatic nerve with radiolabeled aBuTX reveal the axonal transport of nicotinic-like binding sites which are pharmacologically more similar to central nervous system (CNS) nicotinic receptors than to nicotinic receptors on skeletal muscle. It is suggested that these binding sites are destined to become prejunctional nicotinic cholinoceptors. This quantitative autoradiographic method has also been used to evaluate the effects of various toxic agents on the axonal transport of these binding sites.

The effects of organophosphate agents in vitro and in vivo on choline efflux from VPRH have been examined to determine the role of precursor availability in neuromuscular transmission when compromised by AChE inhibition. The neurotoxic organophosphate agents diisopropylfluorophosphate (DFP) and triorthocresyl phosphate decreased choline efflux from VPRH; paraoxon and physostigmine did not alter ACh release. Reduced choline efflux was associated with a frequency-dependent decrease in ACh release. It is postulated that the neurotoxic organophosphate agents interfere with choline liberation via altered phospholipid metabolism.

Subchronic exposure of the rat to DFP resulted in residual alterations in neuromuscular transmission in the isolated VPRH. Negative feedback was present as determined by FC without additional AChE inhibitor in vitro and was antagonized by dTC.

A sensitive chemiluminescent method for picomolar amounts of ACh has been developed and implemented in this laboratory for routine measurement of ACh in perfusate from VPRH. This method is an appropriate replacement for the radioenzymatic assay previously used inasmuch as it is less expensive, safer, and of equal sensitivity (3 pmol).

In conclusion, the results of this investigation support the hypothesis that ACh release at the motor nerve terminal is modulated by local mechanisms capable of acting independently or interdependently of CNS input. These mechanisms appear to be sensitive to the immediate environment of the nerve terminal. One mechanism which appears to be important in the regulation of ACh release is a prejunctional ACh receptor on nerve terminals. This putative cholinoceptor is nicotinic by virtue of its pharmacologic properties, appears to be axonally transported, and operates in a negative

feedback mode. Stimulation of the receptor with nicotinic agonists decreases stimulated ACh release; antagonism of the receptor with nicotinic antagonists increases ACh release. The nicotinic antagonists counter the depressant effects of the nicotinic agonists on ACh release. The negative feedback by excess poststimulus ACh in the junction is dose- and frequency-dependent; its modulatory role is substantial enough to affect muscular contraction in the VPRH. Results with verapamil and calmodulin inhibitors indicate that calcium plays an interdependent role with the prejunctional cholinoceptor in the regulation of ACh release. Prejunctional cholinoceptor activity appears to be separate from precursor control, i.e., choline availability. Acute and chronic AChE inhibition significantly affects negative feedback through the prejunctional cholinoceptor and alters ACh release. Therefore, the cumulative results from this project support the hypothesis that the nerve terminal possesses local mechanisms for modulating ACh release in response to changing physiologic or pharmacologic demands and in the presence of AChE inhibitors.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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## I. Statement of Objectives

This project was designed to test the hypothesis that motor nerve terminals have the capacity to modulate neuromuscular transmission independently of and/or interdependently with central nervous system (CNS) regulation. That motor nerve terminals "fine tune" junctional transmission is a new concept which diverges from the traditionally held view that an equal and fixed amount of transmitter is released into the neuromuscular junction by each nerve impulse. This hypothesis was proposed to rectify numerous anomalies discovered in the laboratory and in the scientific literature, and thus its testing has broadened our knowledge of the functional aspects of neuromuscular transmission under pharmacologic challenge and stress. The concept of a variable gating mechanism for modulating acetylcholine (ACh) output from motor nerve terminals, and thereby muscular response, introduces the exciting possibility that specific pharmacologic agents can be designed to correct functional disorders of neuromuscular transmission that result from certain human disease states or from intoxication by chemical agents and drugs.

The objective of this study was to investigate three mechanisms through which ACh release may be modulated prejunctionally: 1) prejunctional cholinoceptor regulation of ACh release, 2) modulation of ACh release through preconditioning patterns of nerve stimulation, and 3) precursor-induced alterations of ACh release. The relative importance and interrelationships of these mechanisms in comparison to other local or distal regulatory mechanisms of ACh output at the neuromuscular junction were addressed by direct laboratory experimentation.

## II. Introduction and Background

This Final Report covers 2 years of research effort. Experimentation was conducted in all areas of the specific aims of the original proposal, with considerable emphasis on anticholinesterase-induced backfiring in the phrenic motor nerve and prejunctional cholinergic pharmacology. Two extensive Annual Reports[1,2] described the methodology and experimental results in detail and contained tables and figures of nearly all of the data generated. This Final Report represents a summary of that work and will refer to the Annual Reports[1,2] for further detail as needed.

The mammalian model system of neuromuscular transmission used throughout this investigation has been the vascular perfused rat or mouse phrenic nerve-hemidiaphragm preparation (VPRH or VPMH, respectively). The $^{125}$I-alpha-bungarotoxin (aBuTX) binding studies have been performed in rat sciatic nerve because of the anatomy and ease of ligation in situ. ACh release has been measured directly by radioenzymatic assay (REA) and chemiluminescence assays (CLA) and indirectly by either intracellular recording or force of contraction (FC) measurements. The new CLA of ACh has recently replaced the routine REA and the high performance liquid chromatography (HPLC) method investigated earlier in this project.

## III. Results

This section is subdivided into eight parts arranged according to the general focal points of the experimental effort.

### A. Agonist and antagonist interactions with the putative prejunctional cholinoceptor

Our hypothesis states that nicotinic agonists in sufficient concentrations will activate a prejunctional cholinoceptor on the motor nerve terminals and under higher frequencies of stimulation will cause a decrease in the amount of ACh released per impulse. Conversely, a block of the prejunctional cholinoceptor by a nicotinic antagonist will prevent negative feedback and result in an increase in the amount of ACh released per impulse. This hypothesis has been tested by a variety of synergistic methodologies. FC measurements, direct assay of ACh release from the VPRH, and intracellular recording from the neuromuscular junction have been used to assess the effects of nicotinic agonists and antagonists in modulating ACh release through the putative prejunctional cholinoceptor. Nicotine, carbachol, ACh, and suberyldicholine have been used as agonists. Neostigmine (NEO) and related acetylcholinesterase (AChE) inhibitors have been used to raise pharmacologically the poststimulus levels of ACh (the natural agonist) at the neuromuscular junction. Curare, aBuTX, hexamethonium, pancuronium, and edrophonium have been used as potential nicotinic antagonists.

In general, the effects of nicotinic agonists on neuromuscular transmission were in agreement with the stated hypothesis; i.e., transmission was depressed. Suberyldicholine (SDCh, 10 uM), a potent nicotinic agonist, caused a 39% decrease in stimulated ACh release from VPRH as measured by REA. This effect was readily reversible on washout of the drug from the preparation. This depression of ACh release was even more pronounced than the action of equimolar concentrations of nicotine. The action of SDCh corroborates, therefore, studies with carbachol (10 uM) and nicotine (10 uM) in which release was depressed by 23% and 27%, respectively.[2,3] The ability of all three compounds to reduce stimulated ACh release lends support to the theory that a prejunctional negative cholinoceptive modulator is operational at motor nerve terminals. Nonreceptor mechanisms could not be ruled out, however, based solely on these data. Nevertheless, these experiments are particularly persuasive inasmuch as a direct measure of ACh was made via biochemical assay, not via a response by the muscle. Thus, some factors like receptor desensitization may be ruled out. The one complicating factor appears to be the required use of an anticholinesterase inhibitor in the perfused preparation when coupled to the REA or CLA for ACh. NEO or a similar anticholinesterase agent would raise the poststimulus junctional concentration of residual ACh, thus evoking, in theory, a strong negative feedback response even in the absence of an exogenous nicotinic agonist. Thus, our observation of additional "negative feedback" by the nicotinic agonists reveals an apparent wide modulatory capacity within the system.

The actions of the nicotinic antagonists tested on neuromuscular transmission gave strong support to the hypothesis that a prejunctional nicotinic

site functions to modulate ACh release. The nicotinic antagonists, d-tubocurarine (dTC) and aBuTX, were shown to enhance stimulated ACh release from the VPRH (as measured by biochemical assay), as predicted by our working hypothesis.[2,3] dTC showed a dose dependency supporting the hypothesis at low concentrations ($10^{-6}$M-$10^{-8}$M) but having a paradoxical action at high concentrations. At high concentrations ($10^{-4}$M), dTC may block open cationic channels in addition to nicotinic receptors, thus accounting for a block in transmission. aBuTX caused an irreversible action, supporting the hypothesis, but limiting additional work with this compound for the release studies. Experimentation, therefore, primarily focused on low concentrations of dTC ($<10^{-7}$M) as an antagonist to negative feedback by nicotinic agonists. Biochemical assays indicated that 0.1 uM dTC antagonizes the depressive effect of nicotine (10 uM) on stimulated ACh release from the VPRH. Moreover, low doses of dTC ($10^{-7}$M-$10^{-8}$M) antagonize the negative effects of nicotinic agonists on the FC (at 10 and 20 Hz) in stimulated VPRH.[4] It appears that select prejunctional cholinoceptor antagonism may have the potential of raising ACh release in conditions of compromised release. In addition, dTC has been studied by intracellular recording techniques in association with the frequency dependence of negative feedback. These data are discussed in Section D below. Finally, dTC has been employed as a nicotinic antagonist to block AChE-induced backfiring in the phrenic motor nerve (Section F below).

The use of the antagonist dTC has strengthened the theory that backfiring is mediated through prejunctional nicotinic cholinoceptors. These results strengthen the theory that ACh release may be modified prejunctionally by pharmacologic agents and/or during physiologic conditions which create an imbalance in the amount of stimulated ACh release (natural venom or toxin exposure; certain disease states) or hydrolysis of ACh (e.g., AChE inhibition).

In conclusion, the experimental results with the nicotinic agonists and antagonists clearly support the theory that ACh release can be modulated by cholinergic agents. This prejunctional site of action diverges from the dogma traditionally held for only postjunctional sites of action. The apparent competitive interaction of the nicotinic agonists and dTC on ACh release strengthens the hypothesis that a prejunctional nicotinic site exists on motor nerve terminals. Although the physiologic role of this site is open to speculation, it is evident that it is pharmacologically active and manipulatable. The exact mechanism by which this cholinoceptor site modulates ACh release is unknown at the present time.

### B. The neuronal role of calmodulin in neuromuscular transmission

If one were to design a mechanism to modulate ACh release, regulation of calcium entry would be a logical control point. Depolarization-evoked ACh release from phrenic motor nerves requires calcium.[5] Calcium enters the nerve terminal at the time of depolarization of the terminal branches by the invading action potential. The amount of transmitter release appears to be dependent on the concentration of $Ca^{2+}$ entering the cell. In searching for

a rapid prejunctional mechanism to control ACh release, we explored the possibility that the prejunctional receptor could be associated with a voltage-dependent calcium channel. This was accomplished, in part, in the backfiring studies in mouse hemidiaphragm-phrenic nerve (Section F). In addition, this effort included new experimentation to learn the fate of $Ca^{2+}$ once it enters the terminal in response to depolarization. The intraterminal mechanism by which $Ca^{2+}$ influx effectuates transmitter release is not understood. Recent studies reveal that the $Ca^{2+}$ binding protein, calmodulin, may play a role in the excitation-secretion coupling mechanism.[6] In theory, $Ca^{2+}$-calmodulin regulates ACh release by altering cAMP concentrations in the nerve terminal via activation of a $Ca^{2+}$-dependent adenylate cyclase. A transient rise in cAMP levels would activate a protein kinase(s) which mediates phosphorylation of a protein associated with the release mechanism. Inhibition of calmodulin would be expected to decrease this $Ca^{2+}$-dependent increase in cAMP and, thus, to depress ACh output. In contrast, elevating cAMP levels by the activation of adenylate cyclase, the inhibition of phosphodiesterase, or the introduction of dibutyryl-cAMP (db-cAMP) would be expected to antagonize the effects of calmodulin inhibition. In the present study, these theories were partially tested in an isolated neuromuscular model system using four putative calmodulin inhibitors and three cAMP "enhancers," individually or in combination.[2,7]

VPRH preparations from Long-Evans hooded male rats (275-350 g) were used in these experiments. FC was measured via a force transducer during indirect stimulation of the nerve (1.2 V, rectangular pulses, 0.2 ms duration) or by transmural stimulation of the muscle directly (100 V, supramaximal). In 36 additional experiments, the release of ACh was measured in perfusate from the VPRH by CLA[8,9] (described in Section H below). Recovery of released ACh in the perfusate required the presence of the AChE inhibitor, NEO ($10^{-5}$M). These preparations were continuously stimulated at 10 Hz (indirect) for 75 min to establish a steady state control level of ACh release; then drug was introduced for 45 min. Untreated control VPRH's maintained a constant release of $6.52 \pm 0.55$ pmol/min/hemidiaphragm from 60-180 min.

Fluphenazine (FLU), chlorpromazine (CPZ), and trifluoperazine (TFP), neuroleptic phenothiazines with known anticalmodulin activity, induced a decrease in FC via indirect, but not direct, muscle stimulation at $10^{-4}$M. These compounds had no effect on FC at $10^{-6}$M and had only marginal, statistically insignificant depressive effects on FC at $10^{-5}$M. N-(6 aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), a relatively specific calmodulin antagonist, produced a depression in both indirectly and directly evoked FC. Depression of FC by W-7 by direct stimulation was not unexpected since it inhibits calmodulin-stimulated myosin light chain phosphorylation. Washout of these 4 anticalmodulin compounds for 30-45 min results in only a marginal reversibility of less than 10% increase in FC or no reversible change at all.

Three compounds which elevate intracellular cAMP levels were tested separately to determine their effects on normal FC at 0.5 Hz. Forskolin ($10^{-6}$M, n=2; $10^{-5}$M, n=2; $10^{-4}$M, n=1), an adenylate cyclase activator, had no

effect on FC. db-cAMP ($10^{-5}$M, n=2; $10^{-4}$M, n=1) increased FC by 19% and 44%, respectively, during indirect stimulation; direct stimulation provoked similar increases. Theophylline (THEO) ($10^{-4}$M, n=2; $10^{-5}$M, n=2), a phosphodiesterase inhibitor, increased FC by 23% and 42%, respectively, for indirect and, similarly, for direct stimulation. THEO ($10^{-3}$M) kept FC at control levels in the presence of all 4 calmodulin inhibitors ($10^{-4}$M). db-cAMP ($10^{-4}$M) did not significantly antagonize the decreases in FC induced by FLU ($10^{-4}$M) and CPZ ($10^{-4}$M); however, 1 mM db-cAMP antagonized the depressive actions of FLU ($10^{-4}$M) and CPZ ($10^{-4}$M). Forskolin ($10^{-5}$M) completely antagonized the depressive effects of FLU ($10^{-4}$M) on FC.

The stimulated release of endogenous ACh from the VPRH as measured by biochemical assay was significantly decreased by FLU ($10^{-4}$M) and CPZ ($10^{-4}$M). W-7 ($10^{-4}$ and $10^{-5}$M) also depressed stimulated (10 Hz) ACh release by approximately 20-25% as compared to control. THEO ($10^{-4}$M) antagonized the depressive effect of FLU on stimulated ACh release. THEO was not tested with the other compounds. Washout of FLU, CPZ, or W-7 for 30 min failed to reverse the depression. These release experiments were a critical test to prove that the FC data were truly reflective of alterations in ACh release.

A series of intracellular recording experiments were performed in mouse hemidiaphragm to test the "real time" action of FLU and W-7 on neuromuscular transmission.[10] Twitch was blocked by dTC. Endplate potential (EPP) amplitudes were rapidly reduced to near zero by FLU at $10^{-4}$M, but were nearly unaffected or increased slightly by FLU at 5 X $10^{-6}$M. It is noteworthy that the EPP represents the action of quantally released ACh on the postjunctional nicotinic receptors of the myofibril endplate. Thus, it represents an indirect measure of the amount of ACh released. Preliminary calculations indicate that quantal content is reduced; however, these calculations become difficult inasmuch as miniature endplate potentials (MEPP) are also reduced, first by curare and also by the "calmodulin inhibitors," in parallel to the reduction in EPP amplitude. W-7 ($10^{-4}$M-$10^{-5}$M) reduced EPP amplitudes to zero. A 50% reduction in EPP amplitude was observed with $10^{-6}$M W-7. EPP amplitude was unaffected by $10^{-7}$M W-7. Preliminary tests with THEO failed to reveal reversal of the effects of FLU or W-7. Additional experiments clearly demonstrated that the effects of FLU and W-7 were additive to dTC in reducing EPP amplitude. Even in the absence of dTC, FLU and W-7 at the higher dose ($10^{-4}$M) were capable of completely and irreversibly blocking the EPP. This blocking action could have resulted from a significant attenuation of quantal ACh release or a block of the postjunctional nicotinic receptors. Our experimental design could not distinguish between the two possibilities. Future plans involve the local microinjection of ACh to the endplate region to test the EPP-like response in the presence of these so-called calmodulin inhibitors. Nonetheless, our other results predict that the primary effect of FLU and W-7 is to inhibit ACh release.

The results of this multidisciplinary battery of experiments demonstrate that the calmodulin inhibitors FLU, CPZ, and TFP depress neuromuscular transmission by a prejunctional mechanism. W-7 appears to reduce FC by both a prejunctional and a postjunctional mechanism. In support of our hypothesis, the prejunctional mechanism appears to involve an inhibition of

neurotransmitter release, since we have observed a decrease in ACh release in the presence of FLU and CPZ. In addition, we have demonstrated that drugs which increase cAMP levels within the nerve terminal antagonize the calmodulin inhibitors in equimolar or greater concentrations. These results lend support to the theory that stimulus-evoked ACh release is regulated by a cascading pathway involving $Ca^{2+}$-calmodulin-adenylate cyclase-cAMP-protein kinase-protein phosphorylation-ACh release mechanism. These data must, however, be viewed cautiously for two reasons: 1) calmodulin and cAMP may not be directly coupled in the excitation-secretion mechanism for ACh release; thus, we could be indirectly compensating for calmodulin inhibition via another independent modulatory mechanism, and 2) the concentrations of drugs required to induce changes are quite high. Other investigators in the calmodulin area view these concentrations as acceptable in order to get the drug to the intraterminal site of action. Nonetheless, one must consider the possible inhibition of other neuronal enzymes at these high doses or the membrane stabilization or channel-blocking effects of the phenothiazines which would impede cation flux across the depolarized membrane. Furthermore, our results do not establish that the calmodulin system is directly coupled to the depolarization-induced entry of $Ca^{2+}$ nor to the prejunctional nicotinic receptor on motor nerve terminals. Thus, further in-depth experimentation, perhaps in another cholinergic model system and with more specific calmodulin inhibitors, is required to elucidate fully the role of the $Ca^{2+}$-calmodulin in neuromuscular transmission.

### C. $^{125}$I-aBuTX binding sites in sciatic nerve

In neurons, neurotransmitter receptors are synthesized in the cell body and transported to their destined site of action. If prejunctional nicotinic cholinoceptors populate the terminal regions of motor nerves, then these receptors would be expected to be transported from the cell body down the axon to their terminal region. We tested this hypothesis by adapting a radioligand binding method for tracking the axonal transport of "receptor" sites in motor neurons.[1,2,11]

The sciatic nerves of anesthetized male Long-Evans rats (250-300 g) were ligated with silk suture 1 cm below the sciatic notch. Nerve sections (1.5 cm) were removed 0-20 hours later. Eight micron sections were prepared cryostatically and thaw mounted on treated slides in preparation for the binding studies. Autoradiograms were prepared using a modified method of Young and Kuhar[12] by incubating the slides under various treatments with 2.0 nM $^{125}$I-aBuTX (200-400 Ci/mmol; New England Nuclear, Boston, MA). Following a defined incubation and wash period, the slides and photo-emulsion-treated coverslips were stored at 4°C for a month. Autoradiographic grain densities were subsequently determined microscopically under 100X oil immersion. The methodologic details of this quantitative receptor autoradiography have been published.[11,13]

The results of these experiments showed that $^{125}$I-aBuTX binding sites were transported in an orthograde and retrograde time-dependent manner in ligated rat sciatic nerve. $^{125}$I-aBuTX binding to sections of ligated sciat-

ic nerve was saturable, with apparent dissociation constants of 0.97 nM proximal and 0.53 nM distal to the ligature. Accumulation of the binding over time and the displacement of binding by cholinergic drugs were examined. Nicotine, dTC, decamethonium, and atropine displaced binding with affinities comparable to those reported for the toxin binding component in rat brain. These data confirmed the nicotinic nature of the binding sites and revealed binding characteristics more similar to the CNS nicotinic receptor than the skeletal muscle receptor.[13,14] Axonally transported toxin binding sites may correspond to those previously localized to the plasma membrane of peripheral nerve axons and on the terminals of notor neurons. Thus, these results indicate that nicotinic cholinoceptors are axonally transported to the terminals of motor neurons, thus supporting the hypothesis that cholinergic motor nerve terminals contain prejunctional nicotinic cholinoceptors.

We also explored the possibility of using this "receptor" binding method in studying axonal transport in the cholinergic system during toxicologic insult.[15] In brief, our quantitative autoradiographic technique has been utilized to evaluate the effects of various toxic agents on the axonal transport of aBuTX binding sites in rat sciatic nerve. The agents used have been shown elsewhere to produce peripheral neuropathies. Treatment of rats with B,B'-iminodipropionitrile (IDPN) caused a marked reduction in the amount of binding sites, reflected as silver grain densities, accumulating at both sides of a ligature placed around the sciatic nerve for 8 hours in vivo. A similar finding was observed when the nerve was crushed 7 days prior to ligation. In contrast, acrylamide treatment caused a reduction of binding sites accumulating distally to the ligature only. No changes were seen after p-bromophenylacetylurea (BPAU) treatment. We suggest that this technique will be of value in the study of experimental neuropathies induced by chemical agents as a method to quantify the axonal transport of specific proteins.

In conclusion, these $^{125}$I-aBuTX binding studies gave a positive answer to the question regarding the axonal transport of the putative nicotinic receptor as a prerequisite for their existence at the nerve terminal. This work provided fundamental new information on the binding characteristics and cholinergic drug interactions of the axonally transported nicotinic cholinoceptors. Finally, the methodology has been extended to prove its utility in studies of neurotoxicity involving disruption of axonal transport in peripheral motor neurons.

**D. Frequency dependence of negative feedback and ACh release: the action of curare**

Most in vitro studies on neuromuscular transmission employ artificially low frequencies of nerve stimulation (1 Hz), unlike those found in vivo (10-80 Hz or more). This may explain some of the discrepancies found in the literature on cholinergic pharmacology of motor neurons. We designed a series of studies to ascertain the effect of frequency on the release of ACh when challenged by cholinergic agents. The stimulation rates chosen more

closely resemble those found in a physiologically active neuromuscular preparation in vivo. It was of interest to test our hypothesis on prejunctional cholinoceptor feedback under a working situation and/or during stress. An electrophysiologic method using intracellular recording was used in order to obtain a real time assessment of the processes under study.

A driven skeletal muscle (such as the diaphragm during jogging) is required to release a significant amount of ACh in response to 10-40 Hz of continuously fluctuating nerve stimulation. Negative feedback of ACh would be predicted, therefore, as a conservatory and modulatory mechanism locally tuning the activity of the nerve terminal to meet the load. At higher frequencies of stimulation, EPP amplitude would be expected to decrease as less ACh was released. dTC would be expected to have a dose-dependent action on the amount of ACh released by antagonizing the putative prejunctional cholinoceptor. Higher concentrations of dTC should antagonize the negative feedback and flatten the amplitude vs. frequency response curve.

The frequency dependence of neuromuscular block in the presence of dTC was studied in the VPRH preparation by intracellular recording.[16,17] Preparations were stimulated continuously via the phrenic nerve at either 10 or 20 Hz in the presence of dTC. Within 1 min, contraction ceased and conventional intracellular recording of EPPs was accomplished using glass microelectrodes filled with 3 M KCl (5-15 Mohms). Since impalement of muscle cells could not be maintained with intermittent stimulation, preparations were stimulated continuously at either 10 or 20 Hz (holding frequencies), with intermittent stepping to test frequencies (2.5, 5, 10, 20, and 40 Hz) for 2 min. For an experiment at a given holding frequency, mean EPP amplitudes were calculated from a single cell every 3 min. Means were calculated from EPPs which were sampled over a 90-second period with the aid of the Smartscope Waveform Analyzer (T.G. Brandon Corp., Portland, OR). Mean EPP amplitude at a test frequency was determined 30 seconds after the start of that frequency. The mean EPP amplitude during a period at the holding frequency was always determined before the mean at a test frequency. For each test frequency, the mean EPP amplitude relative to the mean EPP amplitude of the immediately preceding hold period was calculated (mean EPP test/mean EPP hold). Data were analyzed by linear regression by least-squares.

In the presence of 0.5 uM dTC, an increase from the holding frequency decreased EPP amplitude; a decrease in frequency increased EPP amplitude. This relationship between neuromuscular block and stimulation occurred at both holding frequencies (10 and 20 Hz). It was also observed that stimulation can be maintained at the holding frequencies for 20 min or more with little change in EPP amplitude. Relative EPP amplitudes decreased linearly with the log of stimulation frequency regardless of whether the holding frequency was 10 or 20 Hz. Furthermore, the slopes of the least-squares regressions of relative EPP amplitude vs. log of frequency were not significantly affected by the holding frequency (-168 when the holding frequency was 20 Hz; -153 at 10 Hz). This log-linear relationship between stimulation frequency and neuromuscular block extended from subtetanic (5 Hz) to tetanic frequencies of stimulation (40 Hz). When the stimulation frequency was less

than 1 Hz, muscle movement made cell impalement impractical at this concentration of dTC.

An additional series of experiments tested the hypothesis that different concentrations of dTC would alter the frequency dependence of neuromuscular block. At both a lower concentration ($2.5 \times 10^{-7}$M, 20 Hz holding frequency) and a higher concentration ($1.5 \times 10^{-6}$M, 10 Hz holding frequency) of dTC, EPP amplitude was less at higher frequencies. The slopes of the least-squares regression line of relative EPP amplitude to log of stimulation frequency were significantly different, the slope being less steep at the higher concentration (slopes: -201 at $2.5 \times 10^{-7}$M; -94 at $1.5 \times 10^{-6}$M). Since the holding frequency does not affect the slope of the regression line, we conclude that increasing concentrations of dTC decrease the frequency dependence of neuromuscular block. This is an important observation (i.e., the flattening of the EPP amplitude vs. frequency response curve) inasmuch as it supports the hypothesis that dTC has a prejunctional action.

In conclusion, EPP amplitude decreased as the frequency of stimulation increased (2.5-40 Hz) in the presence of dTC. This dependence decreased as dTC concentrations increased over the range of $2.5 \times 10^{-7}$M to $1.5 \times 10^{-6}$M. Hence, dTC most likely interferes with the theoretical negative feedback mechanism (e.g., prejunctional cholinoceptors) under stidy. The demonstration of a frequency-dependent dTC block at tetanic frequencies is also consistent with the channel-blocking hypothesis of tetanic fade, but it is evident that this hypothesis does not provide a complete explanation of frequency dependence of neuromuscular block in the presence of dTC. Indeed, several other experimental observations[2,17] are inconsistent with expected consequences of dTC block of postjunctional ACh channels: 1) the frequency dependence of neuromuscular block is not enhanced by increased dTC concentration; 2) low doses of cholinesterase inhibitors do not increase tetanic fade; 3) tetanic fade does not depend upon resting membrane potential; and 4) endplate currents produced by ACh iontophoresed at tetanic frequencies in the presence of dTC do not exhibit fade. Thus, it is evident that the postjunctional channel-blocking hypothesis does not sufficiently explain frequency-dependent neuromuscular block in the presence of dTC. It is concluded that dTC may influence neuromuscular block via a prejunctional action, affecting ACh release. This conclusion is in support of the central theme of this project, i.e., ACh release is modulated via a prejunctional nicotinic cholinoceptor; this process is intimately linked to the frequency of stimulation.

### E. Effect of organophosphate agents on choline efflux from the VPRH and its influence on ACh release

Neuromuscular transmission in the cholinergic motor systems requires an efficient, replenishable, synthetic mechanism for providing neurotransmitter to keep pace with demands. This is particularly true in the phrenic nerve-diaphragm in order to sustain life. ACh synthesis by motor nerve terminals requires an adequate supply of its precursor, choline. This supply is generally abundant through choline in the plasma from the diet and phospho-

lipid metabolism as well as choline in the vicinity of the nerve terminal due to the hydrolysis of ACh by AChE. Precursor availability was examined as a possible mechanism by which ACh release could be controlled, thus explaining preliminary observations with several AChE inhibitors (e.g., physostigmine).

Early experimentation in this project[1] clearly showed that the negative feedback attributed to a prejunctional cholinoceptor site was not linked via a choline availability mechanism.[1,18] The temporal aspects and pharmacologic data were inconsistent with this hypothesis. The frequency-dependent negative feedback which responds to nicotinic agonists and antagonists occurs too rapidly for a choline uptake and/or ACh synthesis mechanism. Tissue levels of choline at the endplate region did not appear to vary significantly during negative feedback, nor did ACh levels predicted if the readily releaseable pool of transmitter was depleted. These observations focused our attention on a fast, perhaps membrane channel-linked process for the prejunctional cholinoceptor mechanism.

These studies also produced interesting, unexpected data on the effects of organophosphate compounds on choline efflux from the VPRH.[19] We have found that diisophopylfluorophosphate (DFP), an AChE inhibitor commonly used in ACh release studies, reduces the rate of endogenous choline efflux from the VPRH. This was determined in the VPRH arranged to measure ACh release except that instead the concentration of free choline in the perfusate was measured. The HPLC method originally developed early in this contract for the measurement of ACh was adapted for the routine measurement of choline.[19] Perfusion of the isolated hemidiaphragm with 10 uM or 100 uM DFP reduced choline efflux by 39% and 69%, respectively. DFP administration to rats (6 mg/kg) also lowered the in vitro release of choline by 33%. Triorthocresyl phosphate (TOCP, 10 uM), a neurotoxic organophosphate agent structurally related to DFP, also induced a significant decrease in choline efflux from the VPRH. However, paraoxon (10 uM) did not alter the efflux of choline from the VPRH. Additional experiments revealed that the rate of ACh release from hemidiaphragm preparations perfused with DFP was significantly lower than the rate of release from preparations perfused with physostigmine, an AChE inhibitor which had no effect on choline efflux. The addition of choline (10-30 uM) to the perfusion medium restored the rate of ACh release from DFP-treated hemidiaphragms but did not further elevate ACh release from physostigmine-treated preparations. These results demonstrate that DFP inhibits choline efflux from the isolated hemidiaphragm and further suggest that, by limiting the availability of choline for ACh synthesis, DFP reduces the rate of ACh release in vitro. It is speculated that DFP and TOCP may inhibit the metabolism of phospholipids containing choline, e.g., phosphatidylcholine. The observation that DFP and TOCP, but not physostigmine or paraoxon, interfere with choline efflux in terms of the delayed peripheral neuropathies (caused by the former, but not the latter) is particularly interesting. Perhaps the neuropathy involves the subchronic alterations of phospholipid metabolism.

In conclusion, choline availability does not appear to constitute the mechanism involved in the rapid negative feedback observed in ACh release in

the presence of nicotinic agonists. The ability of DFP to lower choline efflux from the VPRH and negatively influence stimulated ACh release is thought to be a separate and secondary mechanism which may relate more to the neurotoxicity of organophosphate compounds than to physiologic negative feedback control of ACh release via a prejunctional cholinoceptor.

**F. Antidromic activity (backfiring) in phrenic nerve in the presence of an anticholinesterase agent: the role of ACh**

One method of inducing negative feedback in the VPRH is to raise the poststimulus concentration of ACh within the endplate region of the motor nerve by administering an anti-AChE agent to prevent the hydrolysis of ACh. This procedure also may produce stimulus-induced antidromic activity in the nerve. The motor nerve normally conducts action potentials in an orthodromic direction, leading to transmitter release from the nerve terminal and activation of the muscle fiber. Following cholinesterase inhibition, however, antidromic action potentials (backfiring) can be recorded in the motor nerve. This activity arises both spontaneously and in response to a normal orthodromic volley elicited by electrical nerve stimulation (stimulus-induced antidromic activity or SADA). Since this antidromic activity can be blocked by the nicotinic antagonist dTC, we speculated that SADA may be linked to the prejunctional cholinoceptor under study in this project. A series of experiments were designed to explore the origin of backfiring in an effort to learn more about the operation of the cholinoceptor.

The phenomenon of anti-AChE-induced backfiring has been known since the 1940's.[20] However, no one had resolved the controversy as to whether backfiring was due to the direct action of the anti-AChE agent or to ACh feedback or to ACh-induced $K^+$ release from the myofibers.[21] Our protocol was designed to address this controversy.[22] We employed Clostridium botulinum toxin (BOT) and, later, tetanus toxin (TeT) to block ACh release from the nerve terminal.[23] Antidromic activity was assessed after NEO treatment to determine whether SADA could be elicited in neuromuscular preparations in which cholinergic transmission was virtually blocked with BOT or TeT.

The mouse phrenic nerve-diaphragm preparation has been used in all of the backfiring studies. Female Swiss mice (20-40 g) were anesthetized with chloral hydrate (0.4 mg/g) and the left phrenic nerve-hemidiaphragm was quickly removed and placed in a HEPES-buffered physiologic solution (composition in millimolar concentrations: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid 6.0, NaCl 114.0, KCl 3.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, and glucose 11.0; pH 7.4) which was continuously bubbled with 100% $O_2$ at room temperature (20 $\pm$ 2°C). The hemidiaphragm was then cannulated via the central diaphragmatic vein with polyethylene tubing (PE-10) tipped with a 30-gauge stainless steel tube and perfused with oxygen-supersaturated physiologic solution at a continuous rate of approximately 0.05 ml/min. All experiments were performed at room temperature (20-22°C). The cannula was secured in place with surgical silk and the diaphragm was pinned to a layer of Silastic in a polystyrene chamber. To isolate the electrical activity of the nerve, a small amount of paraffin oil was placed above the solution

which bathed the preparation. Once the diaphragm was cannulated and the electrodes were in place, the preparation was allowed to stabilize for 5-10 min before the start of the experimental procedure. The physiologic solution was driven through the vasculature of the diaphragm by an innovative constant pressure perfusion system designed to provide oxygen to the preparation without the necessity of bubbling the bath. Test solutions were introduced via the cannula from one of four alternative reservoirs under the same oxygen pressure. The reservoirs were connected to the cannulae via a 4-way micro-stopcock (HV D4-F, Hamilton, Reno, NV). The distance between the stopcock and the preparation was minimized (10-15 cm) to reduce dead space. This method also allows for the rapid infusion and washout of drugs.

Stimulation of the phrenic nerve was accomplished by means of a pair of wire electrodes (2-3 mm apart), with a square-wave pulse (0.01-0.1 msec duration, supramaximal voltage) delivered by a stimulator (S-48, Grass Instruments, Quincy, MA). Nerve activity was recorded with a pair of silver wire electrodes (2-3 mm apart) placed distally to the stimulating site, amplified (AC amplifier, Grass model P-16), and visualized on a storage oscilloscope. Permanent traces were obtained on Polaroid film using a Tektronix C-54 camera or Smartscope transient recorder. The stimulating and recording electrodes were immersed in paraffin oil which overlaid the preparation. This method was effective in minimizing the stimulus artifact.

Intracellular glass microelectrodes (10-25 Mohms) containing 3 M KCl were used to record muscle endplate activity from the diaphragm. The EPP's recorded by the microelectrode were led to a unity gain amplifier and displayed and recorded on the oscilloscope. Initial control experiments established a protocol of a 10-min NEO (2 uM) application through the perfusate followed by a 30-min wash period with normal physiologic solution. This method results in reasonably constant levels of backfiring for the entire 40-min experimental period during which the action of blocking agents could be reliably tested. Botulinum toxin treatment was used to block ACh release. Mice were treated with a single intraperitoneal injection of 100 ug ($10^5$ $LD_{50}$) of BOT 40 min prior to dissection. This treatment has been shown to produce a highly effective blockade of quantal ACh release.[24] Diaphragms from these animals were rinsed thoroughly in physiologic solution prior to cannulation. SADA could be elicited in all the phrenic nerves of 47 control preparations tested within 1 min after initial infusion of NEO (2 uM). This activity usually consisted of single and compound action potentials (30-200 uV) lasting for up to 60 ms following the conditioning volley. The levels of backfiring varied from animal to animal but usually remained constant for each preparation for the 40-min experimental period (10-min NEO, 30-min wash). Evoked backfiring was tested every 3 min by stimulating the nerve 3 times, 10 seconds apart. This protocol was adopted to observe the possible effects of drug treatments on a characteristic feature of SADA at room temperature; at stimulation rates below 0.1 Hz, the number of antidromic spikes remains constant with successive stimuli.

Spontaneous antidromic action potentials of low and variable frequency, in addition to SADA, were detected in all the control preparations following NEO administration. These spikes were of small amplitude (50 uV) and their

frequency tended to decline with time through the course of the experiment. Raising potassium levels from 4.5 to 15 mM or higher transiently produced similar, intermittent tiny spikes. Attempts to produce backfiring with elevated potassium failed to yield consistent, pronounced antidromic spikes associated with NEO-induced SADA or the ACh bolus trials. Hence, it is unlikely that extensive potassium release from the myofibers causes backfiring. This is also supported by previous observations in this laboratory in which potassium efflux into the perfusate was measured in the VPRH preparation.[5] Complete AChE inhibition with or without stimulation did not alter the potassium concentrations in the perfusate collected periodically from the VPRH.

Addition of dTC (10 min, 0.1-0.4 uM, n=8) following the 10-min NEO treatment reduced or abolished SADA. This blockade was partially or fully restored during wash. Decamethonium (1-2 uM, n=6) had a similar action to that of dTC, but the muscarinic blocker atropine (10 uM, n=3) was without effect.

In three preparations a small bolus (2.5-4.1 ul, corresponding to a 3- to 5-sec drug delivery time at 50 ul/min perfusion rate) of 1 M ACh was applied to the preparation via the perfusate, following the 10-min NEO treatment. In these experiments it was observed that ACh produced a transient increase in the incidence of spontaneous backfiring followed by a block of SADA. Addition of dTC (2 uM) following NEO blocked all backfiring and prevented the effects of ACh.

Neuromuscular blockade following BOT treatment was confirmed by the virtual absence of MEPPS and the elimination of EPP and twitch in response to low frequency indirect stimulation. However, at high stimulus frequencies (50 Hz), sporadic, small amplitude (2-5 mV) EPPs were elicited. After the addition of NEO (2 uM), EPPs elicited by this method show a prolongation of the repolarization phase, characteristic of AChE inhibition.[25]

Stimulus-induced antidromic activity could not be elicited in the phrenic nerves of BOT-blocked preparations upon addition of NEO (2 uM) in a manner similar to controls (n=8). Furthermore, spontaneous antidromic activity was never apparent in any of these preparations after the addition of NEO. However, backfiring was induced in the BOT-blocked diaphragms by the infusion of a bolus of ACh (after 10-min NEO treatment, n=4). It is noteworthy that more recent work in this laboratory utilizing tetanus toxin indicates that exogenously applied ACh is able to elicit backfiring in the absence of NEO.

The experiments reported here demonstrate that NEO is not sufficient by itself to produce SADA <u>in vitro</u> if cholinergic transmission is blocked with BOT.[23] This is observed with concentrations of NEO which are invariably effective in eliciting backfiring in controls. Previous studies have shown that BOT does not prevent the access of the action potential to the nerve terminal.[26] Our observation that sporadic EPP could be evoked during high frequency stimulation of the nerve confirms this finding in our preparation. Furthermore, BOT appears not to interfere directly with the backfiring

mechanism since antidromic activity was observed in BOT-poisoned preparations upon infusion of ACh. Finally, potassium-induced backfiring appears to differ from ACh-induced antidromic activity. These results are consistent with the hypothesis that released ACh feeds back on a prejunctional cholinoceptor site to induce backfiring in the presence of an AChE inhibitor.

In an attempt to further understand the mechanistic nature of backfiring in the phrenic nerve, we examined the connection of calcium antagonism in vitro. Calcium antagonists such as verapamil and nifedipine are known to decrease the antidromic activity associated with post-tetanic potentiation in the in situ cat soleus preparation. Moreover, since organic $Ca^{2+}$ antagonists can interfere with spontaneous quantal release of transmitter, we tested the hypothesis that, like Clostridium botulinum toxin, organic $Ca^{2+}$ antagonists reduce antidromic activity by interfering with spontaneous quantal transmitter release.

The VPMH was prepared for the measurement of antidromic activity as described above. All drugs were dissolved in standard HEPES-buffered medium and administered vascularly via the cannula. Spontaneous antidromic nerve activity was quantified by counting (5001 Universal Counter Timer, Global Specialties Corp., New Haven, CT) filtered potentials above a set trigger level, usually 50 uV, with the aid of a slope/height discriminator (Frederick Haer, Brunswick, ME). Antidromic nerve activity was also monitored aurally (AM 8 Audio Monitor, Grass Instruments, Quincy, MA) and visually on an oscilloscope; these observations were used to assure the accuracy of the count. NEO (2 uM) was administered for 10 min. Antidromic nerve activity was then monitored for 20 min after NEO was halted. The number of potentials in an 8-min control period (minutes 3-10) was recorded. At the end of minute 10, the preparation was perfused with a second aliquot of control medium with or without 5 uM verapamil (dissolved in coltrol medium in the presence of not more than 0.024% ethanol), and the number of potentials was recorded during a second period (minutes 13-20). For each preparation, the percent of control activity of the second period was calculated. For the investigation on spontaneous quantal transmitter release (MEPPS), the vascularly perfused mouse phrenic nerve-hemidiaphragm was prepared as for the measurement of antidromic activity, and then conventional intracellular recording techniques were utilized. For one endplate, in each preparation, MEPP amplitude, MEPP frequency, and resting membrane potential (RMP) were recorded for 1 min in control medium and for 1 min at 3-8 min after the start of 5 uM verapamil. Before and after verapamil, the mean amplitude of MEPPs was determined for 2 min of recording with a Smartscope II Waveform Analyzer (T.G. Branden, Portland, OR). MEPP frequency was determined by counting the number of MEPPs in 1 min.

Administration of the anticholinesterase agent NEO evoked spontaneous electrical activity that was observed along the phrenic nerve. Potentials of 50-100 uV were recorded, sometimes in apparent bursts of up to 4 potentials. Microscopic examination of the diaphragm indicated that the electrical activity was accompanied by the contraction of a few muscle fibers. This antidromic activity was most likely due to action potentials of indi-

vidual motoneurons which were firing singly or repetitively. Antidromic activity increased transiently after halting the administration of NEO to the preparation. In control medium (without verapamil), the timecourse of antidromic activity following the cessation of NEO was such that the total number of potentials in the second recording period was similar to the control period (87%). Similarly, when ethanol was present during the second period (0.024%, as a control for the ethanol vehicle for verapamil), the amount of antidromic nerve activity in the second period was almost identical with that of the control period (96%, SEM=12, n=4). Verapamil (5 uM) significantly decreased antidromic nerve activity. In contrast, verapamil (5 uM) did not decrease either MEPP frequency or MEPP amplitude (paired t-test); nor did verapamil affect the RMP; the mean (n=5) RMP was -75 mV both before and after verapamil.

Our earlier results[22,23] favor the hypothesis that antidromic nerve activity is due to the direct action of ACh on the motor nerve terminals; however, it has also been porposed that antidromic activity may result from the efflux of $K^+$ from muscle endplates[21] subsequent to the opening of the ACh-activated ion channel. If this were true, then verapamil might decrease antidromic activity by a postjunctional antagonism of ACh. Since verapamil affected neither MEPP amplitude nor RMP at the concentration used here, it is unlikely that a channel-blocking activity accounts for its antagonism of spontaneous antidromic activity. Thus, attention is redirected to a prejunctional site of action.

Inasmuch as both verapamil and nifedipine antagonize antidromic activity and since these compounds are structurally unrelated $Ca^{2+}$ antagonists, verapamil most likely antagonizes antidromic activity by altering nerve terminal $Ca^{2+}$. The observation that decreased $Ca^{2+}$ or increased $Mg^{2+}$ antagonizes antidromic activity also supports this conclusion. Evidently, these $Ca^{2+}$ antagonists have actions independent of their action on spontaneous quantal transmission which affect motor nerve endings. Both quantal and nonquantal release of ACh could be involved in the genesis of spontaneous antidromic activity. and verapamil selectively affects nonquantal release at the concentration studied here. However, this is unlikely. Alternatively, verapamil may be acting at a site which decreases nerve excitability. This latter possibility is consistent with the finding that the $Ca^{2+}$ antagonist diltiazem decreased the amplitude of spinal action potentials elicited by antidromic stimulation of frog ventral roots.[27] Thus, backfiring may be related to modulation of the depolarized state of the nerve terminal, i.e., excitability. Whetheer the putative prejunctional nicotinic feedback receptor is linked to the verapamil site remains to be determined.

In conclusion, backfiring in the cholinergic motor neuron has been shown to require ACh release. The pharmacologic profile of backfiring suggests generation of activity through a prejunctional nicotinic receptor site. It appears that this site is linked at the membrane level to the excitability (or the depolarization) state of the nerve terminal. Although an ionic channel mechanism is likely, our inconclusive studies with the calcium channel antagonists did not identify the precise mechanism of action responsible for ACh-induced backfiring in motor neurons.

**G. Subchronic *in vivo* exposure to DFP: effect on negative modulation of ACh release**

The organophosphate compound DFP has been shown to reduce the FC in a manner similar to NEO and physostigmine via a frequency-dependent mechanism which we believe involves an indirect effect on a prejunctional cholinoceptor. That is, even partial inhibition of AChE at the neuromuscular junction allows the cleft levels of residual ACh to increase after each nerve stimulation. If the frequency of stimulation is high enough (5 Hz and above), the residual ACh in the absence of AChE activity reaches an effective concentration for stimulating, in theory, the prejunctional ACh autoreceptors. Negative feedback would result so that the next volley of nerve stimulation would fail to release the normal amount of ACh, and so on. Our FC data indicate that negative feedback is present in the isolated hemidiaphragm preparations from rats treated subchronically (1, 3, and 5 days) with sublethal doses of DFP. Atropine was administered with each DFP injection as a precaution against postinjection cholinergic distress. Negative feedback was present without the addition of any other compound and was antagonized by dTC ($10^{-8}$M). Negative feedback disappeared if animals were allowed to recover for 48 hrs prior to isolation of the hemidiaphragm. This is an important observation, indicating that moderate, subchronic exposure to an organophosphate agent alters neuromuscular transmission despite the absence of gross motor deficits in the animal. These alterations could lead to a compromise of neuromuscular function under conditions of mild stress or exertion.

**H. Chemiluminescence method for measuring picomolar amounts of ACh in perfusate from VPRH**

Early in this contract we attempted to replace the REA[28] for ACh with a new HPLC method.[1] After considerable technical effort, it was determined that the HPLC did not have the degree of resolution (2 pmol) required for measuring picomolar amounts of ACh in the perfusate collected from the VPRH. In addition, the assay was a long, slow process due to the HPLC column elution characteristics. We successfully employed the HPLC method for the measurement of choline[19] (described in Section E above). However, it was determined that the HPLC was inadequate and inappropriate for our ACh release studies with the VPRH preparations in which only 1 pmol/min is released at rest and not more than 20-30 pmol/min at maximal nerve stimulation.[5]

The expense, radioactive hazards, interassay variability, and time constraints of the REA prompted examination of another sensitive new method for the measurement of picomolar amounts of ACh.[8] This method uses a chemiluminescent reaction to detect choline derived from hydrolyzed ACh.[29,30] The potential of this method was realized after 18 months of preliminary work and methodologic modification.[9]

The CLA for ACh involves three critical reactions: first, ACh in an aqueous sample is converted to choline by AChE (EC 3.1.1.7); second, choline

is converted to betaine and $H_2O_2$ by choline oxidase (ChOx) (EC 1.1.3.17); and finally, $H_2O_2$ is reacted with luminol and horseradish peroxidase (HRP) (EC 1.11.1.7) to generate the chemiluminescence which is electronically measured as an indicator of the amount of ACh originally in the sample. We modified existing extraction/chemiluminescent methods and tailored the entire procedure to the measurement of picomolar amounts of ACh from VPRH perfusates. We compared the CLA directly to the REA to assure the degree of sensitivity and reliability necessary for routine applications.[9]

In brief, the left hemidiaphragms from male Long-Evans hooded rats (Simonsen Laboratories, Inc., Gilroy, CA) weighing 300-440 g were prepared for vascular perfusion[5,32] with a HEPES-buffered medium (pH 7.4) containing in millimolar concentrations: 116 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 11.0 glucose, 6.0 HEPES, 0.010 choline chloride, and 0.010 NEO to inhibit the hydrolysis of ACh by endogenous AChE. Perfusate was collected from the VPRH continuously in 30-min periods, amounting to approximately 1.1 ml of perfusate per period. The preparation was at rest (unstimulated) for the first 30-min period and then stimulated indirectly (1-2 V, supramaximal; 0.2 ms rectangular pulses to the phrenic nerve via a physiologic stimulator, Grass Instruments, SD9) at 10 Hz for 3 hours to give a total of 7 samples. The perfusate samples were centrifuged for 10 min at 1000 X g and 4°C. In order to determine the amount of ACh released during stimulation, aliquots of samples 2 through 6 were taken for both the periodate precipitation-extraction/CLA (2 x 125 ul per sample as duplicates) and the tetraphenylboron extraction/REA (2 x 250 ul per sample). Calibration of the assays was achieved by adding standard amounts of ACh (0, 30, 60, and 90 pmol) to pooled perfusate (samples 1 and 7, and the remainder of samples 2 through 6).

The tetraphenylboron extraction/REA was performed by the method of Goldberg and McCaman[28] as modified by Bierkamper and Goldberg[31,32] for the VPRH. This REA method was used exclusively in the early phases of this contract.

In preparation for the CLA, the periodide precipitation-extraction procedure of Häggblad et al.[30] was used with the following modifications. A 0.4 M sodium acetate-buffered solution (pH=4.0) was used in place of glass-distilled water to dilute the sample aliquots prior to the periodide precipitation step. This substitution improved luminescence. The improvement was not simply a pH effect since luminescence values for extracted ACh samples did not vary with the pH of the sodium acetate solution (pH 3-7). We avoided the use of tritium-labeled ACh as an internal standard, which contributed to luminescence. Tetramethylammonium chloride (6 mg/ml) was substituted for the bromide. Dilute formic acid ($10^{-3}$M, 400 ul) was used in place of dilute hydrochloric acid to free ACh and choline from their complexes with periodide, washing with 2 ml of anhydrous diethyl ether. Additionally, it was discovered that the ether used for washing must be peroxide-free (tested by the procedure of Stewart and Young[33]). Extracted samples were capped and stored in the dark at 0°C for 12-16 hours. Prior to the assay the extracted perfusate samples were first thawed, uncapped, and placed in an open dark area for 1 hour and then recapped. It was crucial to

uncap the thawed samples to allow the last traces of iodine and ether to evaporate. At all times during the assay the extracted perfusate samples were kept on ice in the dark.

The following luminol-enzyme reagent was used for the CLA: 165 ul of ChOx (100 units/ml) in 4.01 ml of 0.2 M sodium phosphate buffer (pH 8.6), 35 ul of HRP (1 mg/ml), and 170 ul of 1 mM luminol. All of the reagents and samples were kept on ice. An aliquot (100 ul) of each extracted perfusate sample was added to a polypropylene tube (8 50 mm). Each tube was placed in the reaction well of the luminometer (Turner model 20e, thermostated to 30°C). Luminol-enzyme reagent (200 ul) was injected, briskly without splashing, into the sample to start the reaction with choline. (The resulting integrated luminescence of this reaction may be used to quantify choline if desired.) After the chemiluminescence from the reaction had decayed to a stable baseline (8-10 min), the endogenous ACh in the sample was converted to choline by the injection of AChE (100 ul, 12.5 units/ml). The integrated luminescence value for 20 seconds of chemiluminescent reaction of the choline liberated from ACh with luminol-enzyme reagent was used to calculate the amount of endogenous ACh in each sample.

The modified CLA procedure provides an accurate and reliable measure of endogenous ACh released by indirect stimulation of the VPRH. This was demonstrated by a direct comparison of the CLA and REA measurements of ACh released over time.[9] A two-way analysis of variance showed that the results from the CLA did not differ from those of the REA. Both the tetraphenylboron extraction/REA and the periodate extraction/CLA methods yielded limits of sensitivity of approximately 2-3 pmol as determined by extracted ACh standards. Standard curves typically had correlation coefficients (by linear regression analysis) between 0.95 and 0.98 for the CLA in the range of 2-40 pmol. Furthermore, the results indicate that there is less variability in values from the CLA than from the REA. Thus, the CLA yields a more consistent pattern of ACh release over time.

Interference by cholinergic drugs was tested in a second series of experiments with the CLA extraction/assay procedure.[9] Perfusate was collected continuously from the VPRH for 3.5 hrs (30 min rest, 3 hrs at 10 Hz), pooled, and then centrifuged. Aliquots (500 ul) of the perfusate were treated with 5-ul aqueous stock solutions ($10^{-3}$M) of drug to give final concentrations of $10^{-5}$M. A 500-ul aliquot of untreated perfusate containing only released, endogenous ACh served as the control. These samples were extracted and assayed by CLA as described above. The frequently used cholinergic drugs ($10^{-5}$M) which we tested did not alter the luminescence of extracted perfusate. These drugs included atropine, hexamethonium bromide, decamethonium bromide, aminopyridine, aBuTX, DFP, nicotine, carnitine, dTC, physostigmine, and carbachol. A one-way analysis of variance of these data indicated a lack of interference by these drugs. We have found that dTC and physostigmine interfere with the CLA under some circumstances. However, these drugs can be removed chromatographically, dTC by the method of Fletcher and Forrester[34] and physostigmine by the method of Molenaar and Polak.[35]

The CLA has a number of advantages over the REA. It has been our experience that about one VPRH in 10 fails to release normal levels of ACh due to a variety of reasons (e.g., nerve damage during dissection).[5] Although it has not been possible to detect a failed preparation until completion of the REA, the CLA detects problems after running only a few samples, thus saving considerable technician time. Moreover, less sample is required for the CLA than the REA so that an assay may be repeated, if necessary. In addition, the time to run the CLA can be halved by pre-reacting the samples en masse with the luminol-enzyme reagent to remove the choline prior to measuring the ACh. This has been adopted as a routine procedure in our laboratory since it saves time and reduces variability even further. The cost of both set-up and routine operation is less for the CLA than the REA. Reagent cost is lower for the CLA when considering the expense of the radioactive compound, scintillation cocktail and vials, radioactive waste disposal, and service contracts on the scintillation counter for the REA. Thus, the CLA is more economical than the REA.

In conclusion, the CLA procedure described in this report provides a method for analysis of ACh in perfusate collected from the rat hemidiaphragm preparation and yields results similar to those achieved with the REA, but at reduced cost and with minimal cholinergic drug interference. Therefore, this method has totally replaced the use of the REA in this laboratory.

## IV. Discussion

The physiology of neuromuscular transmission has been thoroughly dissected by years of electrophysiologic research. However, the pharmacology of the cholinergic motor neurons and associated skeletal myofibers is only partially understood. The present contract has addressed this gap in knowledge with particular emphasis on local mechanisms in the motor nerve terminal which may regulate the amount of ACh release per nerve impulse. These mechanisms have been disclosed by pharmacologic agents and appear malleable by drugs.

A deficiency in many pharmacologic studies involving neuromuscular transmission has been the failure to account for the effects of stimulation frequency. As a consequence, we have been seeking the relative importance of the ACh release control mechanisms under varying conditions of nerve firing and especially in the presence of anticholinesterase agents. Our goal has been to determine the normal function of the mechanisms of regulation and then to study their function under conditions which mimic disease states, demanding physiologic situations, and conditions of neuromuscular toxicity.

The hypothesis that ACh release is regulated by a prejunctional cholinoceptor has been a dominant area of investigation in our laboratory. We find that nicotinic agonists lower the amount of ACh released during nerve stimulation. The depression in ACh release is substantial enough to decrease the force of contraction in the performing muscle, especially at higher frequencies of stimulation (>5 Hz). The ability of low doses of

curare to antagonize the agonist-induced decrease in ACh release lends support to the theory that a prejunctional cholinergic recognition site, a nicotinic receptor, is involved. However, other mechanisms, such as $K^+$ release and depolarization block, must be addressed as well. Our backfiring studies do not support a substantial influence of $K^+$ in a negative feedback scheme within reasonable physiologic bounds. Intracellular recordings do not unveil depolarization block as a predominant mechanism, although it is occasionally and unpredictably observed. Receptor desensitization must also be considered as an explanation for depressed neuromuscular transmission during elevated agonist conditions in the junctional cleft. There is little doubt that ACh receptor desensitization occurs during long-term exposure to residual agonist in the cleft. However, we conclude from our studies to date that postjunctional receptor desensitization does not account for the rapid negative feedback observed during agonist's presence. This was confirmed by intracellular recordings, measurement of FC, and by actually measuring the release of ACh by biochemical assay.

The nicotinic antagonists dTC ($10^{-6}$M) and aBuTX have been shown to increase the stimulated release of ACh and to antagonize the effects of nicotinic agonists on ACh release. However, these agents do not appear to enhance the normal force of contraction of the muscle. To the contrary, aBuTX actually blocks muscular contraction by "irreversibly" blocking the postjunctional nicotinic receptors; curare has similar properties at higher doses ($10^{-4}$M). Low concentrations of dTC block backfiring and agonist-induced negative feedback and yield frequency-dependent neuromuscular blockade consistent with a prejunctional action. Our results suggest that dTC acts via a prejunctional cholinoceptor to modulate ACh release. These laboratory observations confirm the anecdotal clinical experiences of many anesthesiologists working with nondepolarizing blocking drugs such as dTC and pancuronium.

The natural agonist ACh, unlike the many synthetic nicotinic agonists, is rapidly hydrolyzed in the process of neuromuscular transmission. Its prejunctional actions can be unmasked if its half-life in the cleft is increased. The prejunctional action of ACh is frequency- and dose-dependent. We have discovered that stimulation of the nerve must reach 5 Hz before significant negative feedback occurs in the presence of partial AChE inhibition. We take this to mean that the interval between firing must be short enough to induce the accumulation of ACh in the junctional cleft. This negative feedback affects muscular performance and is antagonized by low doses of dTC ($10^{-8}$M). Subchronic exposure of rats to AChE inhibitors results in a residual negative feedback in the phrenic nerves of isolated VPRH. ACh perfused into the cannulated vascular system of the mouse hemidiaphragm results in transient backfiring of the phrenic nerve. Intracellular recordings in the presence of dTC suggest a prejunctional action where ACh feeds back on the nerve terminal; dTC antagonizes this response in a frequency- and dose-dependent manner. Therefore, ACh undoubtedly exerts a prejunctional action which appears to regulate the amount of ACh released on subsequent stimuli. However, the degree of contribution to regulation of ACh under nonstressed <u>in vivo</u> physiologic states or in the absence of AChE inhibition is uncertain. Nonetheless, it is reasonable to assume that

negative feedback becomes operative and significant during systemic anti-AChE intoxication.

A quantitative autoradiographic technique has demonstrated that $^{125}$I-aBuTX binding sites are transported in sciatic nerve in an orthograde and retrograde manner. We postulate that these binding sites represent cholinoceptors destined to become, in part, prejunctional nicotinic receptors. These studies have been expanded to evaluate the effects of various toxic agents on axonal transport of the binding sites. The methodology appears to be valuable in the study of the neuropathology of certain neurotoxicants with regard to the axonal transport of specific protein substances. Future studies may profit from examining negative feedback in the early stages of a chemically induced peripheral neuropathy when it has been determined that the axonal transport of the binding sites is compromised.

The effect of choline on ACh synthesis and release has been investigated. We have shown previously that prevention of choline uptake by the nerve terminal with hemicholinium-3 (HC-3) induces a decline in stimulated ACh release; i.e., ACh synthesis cannot keep pace with demand. Moreover, elevated choline concentrations (30-60 mM) appear by mass action to raise stimulated ACh release from isolated nerve-muscle preparations; i.e., ACh synthesis is enhanced[32]. Furthermore, the rate-limiting step for ACh synthesis appears to be the availability of choline in the nerve terminal. Thus, we have been interested in the role choline plays in negative feedback and in the general modulation of ACh release. Our experiments have shown that choline (up to 60 mM) has no effect on negative feedback induced by NEO in the FC studies. Under normal physiologic conditions, choline is in excess. However, in the presence of DFP or certain chemical substances such as HC-3, choline availability decreases, resulting in a depletion of releasable ACh during high frequency stimulation. This has been demonstrated in the choline efflux studies from VPRH.[19] Neurotoxic organophosphate agents reduce the efflux of choline from the preparation and have a negative influence on ACh release. The non-neurotoxic AChE inhibitors, physostigmine and paraoxon, do not alter choline efflux or change ACh released. We speculate that DFP and TOCP may interfere with phospholipid metabolism such that choline is not made available from its phosphatidylcholine stores. We conclude from our studies that the negative feedback, which is presumably due to the putative prejunctional cholinoceptor, cannot be through a choline supply problem. The negative feedback occurs in milliseconds, not minutes, as would be necessary for a depletion of stores through blockade of ACh synthesis. Thus, we are dealing with two separate but important mechanisms for modulating ACh release. It is possible that during exposure to certain neurotoxic anticholinesterase agents both mechanisms surface; choline availability at the nerve terminal may decrease and residual ACh in the cleft may increase. Both conditions would decrease the amount of ACh released during stimulation and may have a negative influence on neuromuscular transmission, resulting in muscular weakness.

The origin of motor nerve antidromic activity (backfiring) induced by anticholinesterase treatment was examined in the mouse phrenic nerve-hemidiaphragm preparation. Botulinum toxin was used to determine whether back-

firing is due to 1) a direct effect of the cholinesterase inhibitor on the nerve terminal, or 2) an indirect effect via the prolongation of the action of ACh. In previously untreated control preparations, NEO produced spontaneous and stimulus-induced antidromic activity in the phrenic nerve when rapidly introduced into the diaphragm via its vasculature. This activity could be reversibly blocked by dTC and decamethonium, but not by atropine. NEO-induced backfiring did not occur in preparations in which transmitter release was blocked with botulinum toxin. Infusion of a small bolus of a high concentration of ACh following NEO treatment resulted in a short-term increase in the incidence of antidromic activity, followed by block, in both controls and Clostridium botulinum toxin-treated preparations. It is concluded that transmitter release is necessary for the production of backfiring following cholinesterase inhibition since NEO alone does not elicit antidromic activity in botulinum toxin-treated preparations at concentrations which are effective in controls. Our results support the hypothesis that the effects of NEO on the motoneuron terminal are mediated by the prolonged action of ACh that occurs with inhibition of ACh.

The studies on antidromic firing lend support to the presence of cholinergic recognition sites on motor nerves. These data are supported as well by the $^{125}$I-aBuTX binding sites found to be transported in sciatic nerve. Although backfiring per se is not generally viewed as a normal physiologic means of regulating ACh release, it may play a role in abnormal conditions such as anti-AChE poisoning. Retrograde action potentials would collide with incoming impulses and prevent depolarization of the nerve terminal; i.e., this would prevent ACh release. On the other hand, backfiring could occur in one branch of a motor unit and spread orthodromically at branch points to other branches. This, in effect, would increase ACh release and perhaps fasciculations within the muscle bundle. These studies also promote the consideration of older theories concerned with the role of $K^+$ in the environment surrounding the nerve terminal. It is believed by some investigators[21,36] that SADA may be caused by excessive $K^+$ release (by muscle cells?) so that the nerve terminal would become depolarized and activate the first node of Ranvier. This would evoke a retrograde action potential. Our preliminary data fail to support this $K^+$ theory as the cause of the backfiring induced by the anti-AChE treatment. Instead, a prejunctional nicotinic cholinoceptor mechanism is implied by the data.

The calcium antagonist verapamil decreased spontaneous, NEO-induced backfiring, implicating the need for calcium in the process. It is likely that calcium either plays a role in the amount of ACh released or modifies the membrane potential (and thus excitability) of the nerve terminal or both. This would influence backfiring as we have shown experimentally. Whether a calcium mechanism is directly linked to the putative prejunctional nicotinic cholinoceptor is uncertain.

Additional studies with calmodulin inhibitors lend further support to the role of calcium in modulating ACh release. A test of an hypothesis based on the $Ca^{2+}$-calmodulin-adenylate cyclase-cAMP-protein kinase pathway confirms that ACh release can be modified by antagonizing $Ca^{2+}$-calmodulin. Experiments with theophylline and forskolin suggest that calmodulin is

linked to cAMP which, via a protein kinase, ultimately modifies ACh release. Stimulus-induced calcium entry therefore plays a role in neurotransmitter release. It is conceivable that the prejunctional cholinoceptor is linked to the control of calcium entry since we have shown that the negative feedback is too fast for a mechanism involving ACh synthesis. Whether this link is via the calmodulin pathway is uncertain. Taken together with the backfiring results, these data strongly support the hypothesis that a local calcium regulatory mechanism modulates ACh release.

A CLA coupled to a periodate extraction method has been developed for the measurement of ACh release from the vascularly perfused rat phrenic nerve-hemidiaphragm preparation. A direct comparison of the CLA with an established REA for ACh demonstrates that the two assays are quantitatively similar and yield equivalent limits of sensitivity of approximately 2-3 pmol. The periodate extraction/CLA method is routinely less variable and more reliable than the tetraphenyl-boron extraction/REA method. Cholinergic drug interference with the CLA is minimal. The absence of radioactivity and the reduced cost of the CLA make this assay method an attractive alternative to the REA. Consequently, the CLA method is an appropriate replacement for the REA method.

In conclusion, the results of this investigation clearly demonstrate that ACh release can be modulated by local prejunctional mechanisms. These mechanisms include a prejunctional nicotinic cholinoceptor, precursor availability, and probably nerve terminal excitability as modified by ionic chan nel activity at the membrane level. These mechanisms are dependent on nerve terminal activity as regulated by stimulation rates. All of these mechanisms are disturbed by AChE inhibition induced by the neurotoxic anticholinesterase agents, resulting in a compromise in neuromuscular transmission and, therefore, performance. Finally, this investigation has given conclusive pharmacologic evidence that many cholinergic agents have prejunctional actions in addition to their traditionally accepted postjunctional effects on neuromuscular transmission. This new knowledge will be valuable in explaining drug- and toxicant-induced alterations in ACh release and for devising new therapeutic measures for counteracting irregularities in neuromuscular transmission.

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These publications directly resulted from funding of this contract. All of the work has been published or submitted for publication except for one manuscript (currently in preparation) on the prejunctional pharmacology of the cholinergic agents investigated.


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